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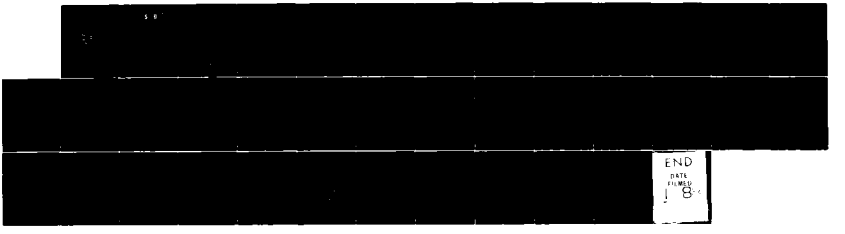

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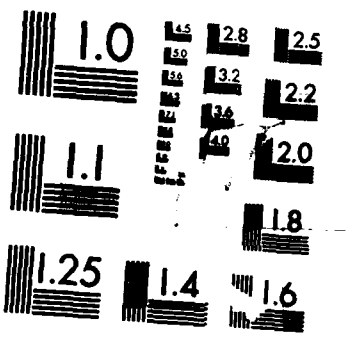

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ENTROPIC ELASTOMERIC FORCE IN PROTEIN STRUCTURE/FUNCTION

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ABSTRACT

Briefly noting earlier studies on the polypentapeptide of elastin, $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$, and on elastin, it is emphasized that entropic elastomeric force can be exhibited by non-random, anisotropic polypeptide systems and therefore that entropic elastomeric force does not necessarily result from isotropic random chain networks as required by the classical theory of rubber elasticity nor does it result from solvent entropy effects as deduced from the slow loss of elastomeric force on thermal denaturation. Instead entropic protein elasticity can be the result of internal chain dynamics, specifically of librational processes that become damped on chain extension. This new mechanism of entropic protein elasticity allows for an understanding not only of elastin but also of the passive tension of striated muscle, of the voltage-dependent interconversion between open and closed conductance states in the sodium channel of squid nerve, and of protein elastic forces producing strain in a substrate bond during enzyme catalysis.

Because entropic elastomeric force develops as a result of an inverse temperature transition, it becomes possible to shift the temperature of the transition to higher or lower temperatures by decreasing or increasing, respectively, the hydrophobicity of the elastomeric polypeptide chain. In warm blooded animals this allows for biochemical modulation of the relaxation or development of entropic elastomeric force by an enzymatically modulated decrease or increase of the hydrophobicity, as for example, by phosphorylation or dephosphorylation of the elastomeric polypeptide chain. This understanding provides a mechanism for modulating protein function, whether for example enzymatic or channel, a mechanism for the remarkable reversible structural processes that attend parturition, and a mechanism for the connective tissue anomalies of wound repair and environmentally induced lung disease.

INTRODUCTION:

Presently recognized as the primary elastomeric protein of warm-blooded animals is elastin; it is the second most prevalent protein in the extracellular matrix with only collagen being more common (1). The nature of the elastomeric force was demonstrated by Hoeve and Flory in 1958 to be dominantly entropic in origin (2). This is an important statement as it provides an understanding of the durability of elastin where single elastin fibers can last the lifetime of an individual which in the vascular system means undergoing more than one billion stress/strain cycles. That elastin is a dominantly entropic elastomer was reaffirmed by Hoeve and Flory in 1974 where they continued also to insist that "A network of random chains within elastin fibers, like that in a typical rubber, is clearly indicated" (3). This perspective has dominated thinking with respect to protein elasticity for nearly three decades and remains a staunchly-held perspective (4-11). Accordingly the insistence that entropic elastomeric force requires a random network of chains has precluded application to protein systems known to be non-random chain networks.

Studying the molecular structure and function of the polypentapeptide of elastin - the most striking primary structural feature of elastin (12), occurring within the longest sequence between cross-links, a sequence twice as long as any other possible elastomeric sequence between cross-links (12,13) - this Laboratory has demonstrated a new mechanism of entropic elasticity for the polypentapeptide of elastin and has demonstrated its applicability to the elastin fiber as a whole (14,15). The mechanism derives from internal chain dynamics and is called the librational entropy mechanism of elasticity. In this report the new mechanism of entropic elasticity is considered relative to other protein systems where elastomeric force is implicated but where the proteins cannot be described as random chain networks.

In particular the identification and possible origins of entropic elastomeric force are briefly considered. The applicability of internal chain dynamics, i.e. librational processes, to protein elasticity as newly understood in elastin is extended to an understanding of the passive tension in muscle, of changing conductance states of channels, and of enzyme mechanisms. Furthermore the relevance of structural transitions to and from the elastomeric state is considered in regard to elastogenesis, to wound repair and fibrotic lung disease and to processes attending parturition and their reversal, that is, cervical ripening and pubic ligament formation.

II. POSSIBLE ORIGINS OF ENTROPIC ELASTOMERIC FORCE IN PROTEINS

Elasticity, of course, is the property whereby a material resists and recovers from deformation. The elastomeric force, f, can be considered to be comprised of two components: an internal energy component, f_e, and an entropy component, f_s, i.e.

$$f = f_e + f_s \tag{1}$$

Following Flory and colleagues the relative magnitudes of the internal energy and entropy components can be determined by means of thermoelasticity studies (16). In these studies the elastomer is extended to a fixed length and the elastomeric force is measured as a function of temperature. A plot of ln[f/T(°K)] versus temperature allows evaluation of the f_e/f ratio, i.e.

$$\frac{f_e}{f} = -\bar{T}\left(\frac{\partial \ln(f/T)}{\partial T}\right)_{P,L,eq} - \frac{\beta_{eq} T}{\alpha^3 (V_i/V) - 1} \tag{5}$$

where the experiment is carried out at constant pressure, P, with the elastomer at fixed length, L, and with the elastomeric matrix in equilibrium, eq, with the solvent. The second term in Eq. 2 is a correction term allowing the analysis to

procede at constant pressure rather than constant volume, and in equilibrium with solvent rather than at constant composition (17). In this term $\beta_{eq} = (\partial \ln V/\partial T)_{p,L,eq}$ is the thermal expansion coefficient; α is the fractional increase in length; and V_i and V are the elastomer volumes before and after elongation. This correction term is of the order of 0.1 for elastin (18) and also for the polypentapeptide of elastin (19). In Figure 1 are thermoelasticity studies for elastin and for the polypentapeptide of elastin where particularly for the latter the near zero slope argues for a dominantly entropic elastomeric force (20). On changing the solvent to ethylene glycol-water, 3:7 by volume, the rapid rise in elastomeric force is shifted to lower temperature and the near zero slope becomes more apparent for elastin (unpublished data, 2,3). Furthermore a near zero slope for elastin has been found in triethylene glycol (10). Thus elastin and the polypentapeptide of elastin are considered to be dominantly entropic elastomers.

A. The Classical Theory of Rubber Elasticity for Random Chain Networks

The classical or statistical theory of rubber elasticity holds that entropic elastomeric force derives from random chain networks (21-23). At rest the network is described as being comprised of a random distribution of chain end-to-end lengths. This is the highest entropy state. On stretching the distribution of end-to-end lengths is displaced from that of highest entropy. This decrease in entropy provides the resistance to deformation and the driving force for recovery. A representative distribution of chain end-to-end lengths is given in Figure 2 where W(r) is the probability distribution of the end-to-end lengths, r, in nm. In this theory the f_e/f ratio is given by $\frac{d \ln \langle r^2 \rangle_o}{dT}$ where $\langle r^2 \rangle_o$ is the mean square end-to-end chain length.

B. Solvent Entropy

When the elastomer is comprised of hydrophobic groups that become exposed to polar solvents such as water on extension, several workers -- Weis-Fogh and Andersen (24), Gosline (25,26), and Gray, et al. (27) -- have suggested that the formation of clathrate-like water surrounding these exposed hydrophobic groups constitutes a decrease in entropy that would provide an entropic restoring force.

C. Internal Chain Dynamics: Librational Process

Another source of decrease in entropy on extension has been derived from studies on the polypentapeptide of elastin (14,15,28-31) but it is an entirely general mechanism. It asserts that chain segments within a bulk matrix have freedom to undergo rocking motions. Since the chain segments in the dense, cross-linked bulk matrix will be essentially immobilized at their ends, motion occurs by rotation about one bond being paired with compensating rotations about one or more other bonds such that rocking motions or librational processes occur. On stretching these librational motions become damped. This has been termed the librational entropy mechanism of elasticity (29).

III. ELASTOMERIC PROCESSES IN PROTEIN SYSTEMS

A. The Polypentapeptide of Elastin

As shown in Figure 1A, when the polypentapeptide of elastin is γ-irradiation cross-linked at a concentration of about 40% peptide, 60% water by weight, the resulting elastomer exhibits dominantly entropic elastomeric force above 40°C. On raising the temperature from 20° to 40°C, however, there is a dramatic development of elastomeric force. This development of elastomeric force has been demonstrated by five independent physical methods--nuclear magnetic resonance structural and relaxation studies, dielectric relaxation studies,

circular dichroism studies, microscopic characterization, and composition studies -- to correlate with development of molecular order, that is, to correlate with an inverse temperature transition (14,19,32). In the 20° to 40°C temperature range development of molecular order correlates with development of elastomeric force. That the entropically elastomeric state above 40°C is an ordered state is further demonstrated by thermal denaturation followed by circular dichroism (19), by extrusion of water (15,19) and most directly by the slow loss of elastomeric force and of elastic modulus (15,33), all demonstrated by heating at 80°C. As the elastomeric state is not a random chain network and since at 80°C destructuring of clathrate-like water would occur with time constant of the order of nanoseconds or less whereas the loss of elastic modulus at 80°C occurs with a half-life of days, the entropic elastomeric force must be due to internal chain dynamics.

The proposed elastomeric structure of the polypentapeptide of elastin is given in Figure 3 (31,34, 35, 28) and the effect of stretching on the damping of the librational motions is shown in Figure 4 (29). The regularly repeating structure of the polypentapeptide provided the opportunity to demonstrate unequivocally that entropic elastomeric force occurs on formation of a regular non-random structure. One of the particularly interesting demonstrations is provided by dielectric relaxation studies (36). At 20°C where there is minimal elastomeric force, the real part of the dielectric permittivity in the 1 GHz to 1 MHz frequency range exhibits a monotonically increasing curve. This is shown in Figure 5. As the temperature is raised and elastomeric force develops, there develops a localized Debye-type relaxation centered near 20 MHz. This has been assigned to a peptide librational mode (14,36). The intensity at 40°C, $\Delta\varepsilon = 70$, and the localized nature of the relaxation require a regular non-random elasto-

meric state and the relaxation identifies a backbone (peptide) librational mode that is directly contributing to the high entropy of the relaxed state. While the phenomenology enumerated above require setting aside the random chain network analysis and require the elimination of solvent entropy as a consideration, this experiment allows direct observation of the responsible internal chain dynamics. This is the remarkable contribution of the polypentapeptide of elastin.

B. The Elastin Fiber

In the case of the elastin fiber three of the five physical methods, utilized to demonstrate that increase in elastomeric force in the below 40°C temperature range correlates with increase in molecular order in the polypentapeptide, have been applied to elastin, to the precursor protein, or to a chemical fragmentation product of elastin. Those physical methods are microscopy (37-40), dielectric relaxation (41) and circular dichroism (42). Furthermore thermal denaturation has been directly observed on elastin, as on the polypentapeptide of elastin, by following the slow loss of elastomeric force in a thermoelasticity study and the slow loss of elastic modulus monitored by stress/strain curves at 37°C which resulted from heating at 80°C (15,33). Therefore the entropic elastomeric force exhibited by this protein is not due to a random chain network nor is it due to the formation of clathrate-like water structures, rather it too must derive from internal chain dynamics. It may be noted that the slow thermal denaturation is in the practical sense irreversible in water. Here again the internal chain dynamics can, with the insight of the studies on the polypentapeptide of elastin and with awareness that the most prominent sequence between cross-links is where the polypentapeptide resides, be directly observed by dielectric relaxation studies on α-elastin (the chemical fragmenta-

tion product of elastin) in the 1 GHz to 1 MHz frequency range as shown in Figure 6 (41). While the intensity of the relaxation is less, as expected with the polypentapeptide being a fractional component of α-elastin, a relaxation is again observed near 20 MHz.

C. Elastomeric Filaments of Muscle

Studies of Maruyama (43,44) and of Wang (45,46) have resulted in the isolation of a several million molecular weight elastic protein from muscle. Efforts to characterize this protein microscopically have demonstrated the protein to be filamentous (43). This protein becomes a possible explanation for the passive tension of muscle and for the residual passive force exhibited when the sarcomere length has been extended beyond the point where the thick and thin filaments no longer overlap. Microscopic studies on pulled fibers have led to the identification of long narrow filaments either connecting the thick filaments to the Z lines or directly running from Z line to Z line (47,48). Consistent with an effort to understand elastomeric force in terms of random networks, it has been suggested that the stretching itself causes the filaments to form from a gel state (see discussion following 47). Consistent with the random chain network theory of entropic elasticity, efforts are made to understand elasticity in terms of an isotropic gel state rather than in terms of the anisotropic filaments observed microscopically on the pulled fibers and observed microscopically for the isolated elastic protein of muscle. With internal chain dynamics having been demonstrated to be the source of entropic elastomeric force, however, it now becomes possible to understand durable non-random, anisotropic elastomeric filaments and thereby to accept the microscopic observations of the isolated elastic protein of muscle and of the pulled muscle fibers.

D. Interconversion of Sodium Channel Conductance States

One of the very challenging aspects of biology to a physical chemist is understanding the molecular structure and mechanisms of ion selective, voltage dependent transmembrane channels. The conductance state -- open, closed, refractory -- depends on the transmembrane potential. It is of fundamental interest, for example, to understand what structural changes and processes result in changing the conductance state. This issue has been addressed in an interesting way by Rubinson (49) who modelled the sodium channel opening/closing equilibrium of squid nerve "as a charged region of a macromolecule moving under the influence of the applied field and confined elastically by interconnection with other masses." The result was the characterization of the mechanical properties of the polypeptide chain segment which controlled the gating process as rubber-like with an elastic modulus in the range of that of elastin. Taking the elastic modulus to be 5×10^6 dynes/cm^2 as for elastin, the ratio of the cross-sectional area to length (-400A) of the connecting chain segment would not be unlike that of the polypentapeptide β-spiral in Figure 3. This is not to imply in any way that a β-spiral like that of the polypentapeptide of elastin actually exists in the sodium channel but rather to emphasize that internal chain dynamics and specifically librational processes rather than random chain networks would be required to understand this elastomeric process.

E. Enzyme Mechanisms

Several spects of enzyme mechanisms may involve entropic elastomeric forces within the protein, for example, the structural rearrangements resulting from the binding of an allosteric effector (50), induced fit elements of substrate binding (51) and the catalytic process itself. In the former two processes it is apparent that binding to the surface of a viscoelastic protein

could result in compressional or extensional damping of librational motions within proximal regions of the active sites. In addition the catalytic process itself has been considered in terms of elastic forces. Recalled for example is the elastomeric "rack" of Lumry and Eyring (52). A recent elegant description of this element of enzyme catalysis has been presented by Gavish (53) in an exposition of "molecular dynamics and the transient strain model of enzyme catalysis." With emphasis on the visoelastic properties of proteins (54), Gavish described a detailed model for stress and stain in the enzyme-substrate complex. The protein exerts an elastic force on the scissile bond of the substrate resulting in a strain that contributes to the potential energy required for bond cleavage. An effective means of increasing the rate of the catalytic process would seem to be to employ an entropic elastomeric force to induce strain in a substrate. Gavish states (53) "factors that dominate structural mobility in proteins should affect enzyme catalysis." On the basis of the new understanding of entropic protein elasticity it might be said that factors that modulate entropic elastomeric force should modulate enzyme catalysis. For entropic elastomeric force as demonstrated by the polypentapeptide of elastin, it is not mobility per se but rather it is mobility arising from a regularity of structure that gives rise to force capable of inducing significant strain. As shown by the NMR-derived rotational correlation times (15), the mean mobility of the peptide moieties is greater at 25°C before the inverse temperature transition than at 37°C after the inverse temperature transition, yet the entropic elastomeric force is minimal at 25°C and dramatically increases until 37°C (15). Thus it is not motion per se but the nature of the motion. In the dielectric relaxation studies at 25°C there is no localized relaxation in the 1 GHz to 1 MHz frequency range, but as the temperature is raised to 40°C, there develops in concert with the development of elastomeric force an intense, Debye-type

relaxation near 20 MHz indicating motion within a regular structure (36). Thus it is coherent motion, e.g. a librational mode, within a regular structure that gives rise to entropic elastomeric force. This provides for an anisotropic structure capable of producing a strain in an enzyme substrate by means of an entropic elastomeric force.

IV. MODULATION OF TRANSITIONS IN THE ELASTOMERIC STATE: TURNING ENTROPIC ELASTOMERIC FORCE ON AND OFF

In the preceding discussion of elastomeric processes in protein systems it was generally the elastomeric state itself that was considered but the modulation of the transition to and from the elastomeric state can be an effective means of turning on and off an entropic elastomeric force. The modulation can be biochemical and it can be involved in such disparate processes as the modulation of enzyme catalysis, wound repair, the destruction of elastic tissue in environmentally induced lung disease, and relaxin induced cervical ripening and pubic ligament formation attending parturition and their reversal.

A. Elastogenesis

Before addressing the more biomedical issues, it is necessary to consider the implications arising from the fact that, for elastin and the polypentapeptide of elastin, elastogenesis arises out of an inverse temperature transition and is therefore dependent on the hydrophobicity of the chains which are to constitute the elastomer. Generally elastogenesis of elastin has been considered to be the physical process of fiber formation but as will be seen below it is simultaneously fiber formation and the development of elastomeric force. This is not possible within the constraints of the classical theory of rubber elasticity requiring as it does random chain networks because the formation of an isotropic random chain network could not result in the formation of anisotropic fibers. Once the random chain network perspective is set aside, it

becomes apparent that modulation of elastomeric force in homoiothermic animals can be achieved by shifting the temperature range in which the inverse temperature transition occurs.

1. Effect of Changing the Hydrophobicity

Using the polypentapeptide of elastin as the model elastomer, analogs can be prepared in which the hydrophobicity of the repeating unit is changed. Three physical characterizations can be compared: (1) the temperature profile for aggregation, which is actually the temperature profile for fiber formation, 2) the temperature dependence of conformational change followed by circular dichroism, and 3) the temperature dependence of elastomeric force of the γ-irradiation cross-linked analog which has been stretched to a fixed length at 40°C. As shown in Figure 7, these transitions occur near 30°C for $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$, the polypentapeptide of elastin. When the hydrophobicity of the repeating unit is increased as in $(Ile^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$, the Ile^1-polypentapeptide, the temperature of the transition, as followed by all three means shifts to lower temperature by some 20°C to near 10°C (55). When the hydrophobicity of the repeating unit is decreased as in $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Gly^4)_n$ where the Val^4 residue has been deleted, the temperature of the transition shifts some 20°C to higher temperature to near 50°C (56). These shifts are proportional to the hydrophobicity of the repeating unit as estimated by the Nozaki and Tanford (57) and the Bull and Breese (58) scales. This reaffirms the transition to be an inverse temperature transition, with a temperature inversely proportional to the hydrophobicity of the repeating unit. It is to be emphasized that the transition for the development of elastomeric force follows the hydrophobicity shifts; this further reaffirms development of elastomeric force to be the result of an inverse temperature transition leading to increased order for the elastomeric state (55,56).

2. Effect of the Transition on the Length of the Elastomer

The steepness of the curve for the development of elastomeric force of the Ile^{1}-polypentapeptide near 10°C (see Figure 7C) is the result of matrix shortening and the fact that this sample had been stretched to 40% elongation at 40°C whereas the other samples had been stretched to 60% elongation at 40°C. As reflected in the temperature profiles of aggregation, the noncross-linked polypeptide is soluble in all proportions at a temperature below the onset of the inverse temperature transition (19). This means that the cross-linked elastomers would dissolve on lowering the temperature below the transition if it were not for the cross-links. Instead of dissolving the cross-linked polypeptides simply swell to the limit allowed by the cross-links and by the structural transition. This results in remarkable changes in the length of the cross-linked matrix as shown in Figure 8 where the length is measured as a function of temperature under zero load (59). For 20 Mrad cross-linked polypentapeptide, the length of a strip of matrix increases 250% as the temperature is decreased from 40° to 20°C. Elastin shows analogous but less dramatic lengthening; a classical rubber such as latex, of course, shortens on lowering the temperature under zero load.

3. Biochemical Modulation of Hydrophobicity, i.e. of Transition Temperature

Instead of decreasing the temperature to relax the elastomeric force, it is possible to modify enzymatically the hydrophobicity of the elastomeric polypeptides and thereby to shift the temperature of the inverse temperature transition. This shift in temperature of the inverse temperature transition has been demonstrated with the enzyme prolyl hydroxylase. As shown in Figure 9 when the polypentapeptide is exposed to prolyl hydroxylase with the

resulting hydroxylation of some of the Pro residues, this decrease in hydrophobicity causes the temperature profile for aggregation (60), equivalently for fiber formation and for elastomeric force development, to shift to higher temperature. This shift occurs with only about one Pro in ten hydroxylated; this is only one hydroxylation in fifty residues. Thus enzymatic prolyl hydroxylation with a sample of X^{20}-PPP held extended at 37°C should result in a decrease in elastomeric force when held at constant length and an elongation of the sample when maintained at a constant force.

While hydroxylation is an irreversible process, it becomes a trivial conceptual step to consider an elastomer with occasional serine or threonine residues that could be phosphorylated by a kinase causing the elastomer to extend, i.e. to relax, and that could be dephosphorylated by a phosphatase causing the elastomer to shorten and elastomeric force to again develop. It is suggested that such processes could be involved in the relaxin induced cervical ripening and interpubic ligament formation and their reversal after parturition. Phosphorylation of enzymes and other proteins such as channels could be expected to have analogous effects on polypeptide segments capable of exerting entropic elastomeric force.

B. Biomedical Relevance

1. Wound Repair

In scar tissue there is a preponderance of collagen fibers with few or no elastin fibers (61). In optimizing wound repair which involves sewing the breach together with collagen fibers, high levels of prolyl hydroxylase occur. Hydroxylation of proline residues in collagen is necessary for release of collagen from the cell; it is required to stabilize the collagen triple stranded helix, and it protects collagen from non-specific proteolysis (see

references within 62). The same enzyme hydroxylates proline residues in tropoelastin, the single precursor protein of elastin fibers. Based on the shift to higher temperatures of the temperature profile for fiber formation of the polypentapeptide of elastin (see Figure 9) that results from prolyl hydroxylation, this decrease in hydrophobicity of tropoelastin would be expected to have a similar effect. The result would be less elastic fiber formation and the fiber formed would be in a more nearly relaxed state and unable to provide an appropriate entropic elastomeric restoring force. This has been demonstrated in cell cultures of aortic smooth muscle cells induced to high levels of hydroxylation by the addition of ascorbic acid required by prolyl hydroxylase (63).

2. Environmentally Induced Lung Disease

In environmentally induced lung disease, such as pulmonary emphysema, the elastin fibers are fragmented and dysfunctional. When the lung is challenged by toxic substances, it is proposed that the ensuing repair response results in the elaboration of high levels of prolyl hydroxylase. The consequence of over-hydroxylation of tropoelastin would limit elastin fiber formation; those fibers that did form would be able to exert a more limited elastomeric function because of the shift to higher temperature of the inverse temperature transition; and it is not unreasonable to expect that the poorly formed fibers would be more susceptible to proteolytic degradation (62). In general any process, such as inhalation of cigarette smoke, that resulted in oxidation of the elastomeric chains in elastin would cause a loss of elastic recoil.

3. Events Attending Parturition and Their Reversal

Interpubic Ligament Formation: There are remarkable deformations and restoring forces attending and following parturition. In mice and guinae pigs (64,65) and in some women there is the development of an interpubic ligament in the days prior to delivery. In mice, for example, the pubic symphysis

is normally less than 2 mm in width. In the days before delivery an interpubic ligament develops that becomes 5 to 6 mm in length allowing for enlargement of the birth canal. By the third or fourth day after delivery the gap between the pubic bones is drawn back to 2 mm (65). What connective tissue processes could allow this elongation, and then within the time period of a few days what restoring forces could result in the shortening? The above mentioned biochemical process of decreasing the hydrophobicity by phosphorylation could lead to lengthening by shifting of the temperature range of the inverse temperature transition for the development of elastomeric force to higher temperature. The result would be a biochemically controlled relaxation of elastomeric force. Subsequent removal of the phosphate moieties by phosphatases would result in a restoration of elastomeric force and a shortening of the elastomer. Interestingly the shortening from about 5 mm to 2 mm is similar to the shortening of the cross-linked polypentapeptide, seen in Figure 8, on going from the relaxed state at 20°C to the elastomeric state at 37°C. A 20°C increase in the temperature range of the inverse temperature transition by decreasing hydrophobicity due to phosphorylation could result in the lengthening and then dephosphorylation could return the transition temperature to its normal physiological range being completed as it is just at body temperature.

Cervical Ripening: The relaxing and softening of the cervix is referred to as cervical ripening. This occurs in the hours preceding delivery and is thought to be under the control of the hormone relaxin (66-68). Here one could employ elastin fibers as considered for the interpubic ligament formation. However, if uterine smooth muscle fibers contained elastomeric filaments as observed in striated muscles, then phosphorylation and dephosphorylation of intracellular elastomeric filaments could readily be considered as a potential mechanism. This is a particularly attractive hypothesis as the mechanism of

action of relaxin is considered to involve the activation of kinases and phosphatases in a time dependent manner (69). Once such a hypothesis is raised involving uterine smooth muscle cells it is natural to inquire whether such a process could be operative in vascular smooth muscle cells and be relevant to some forms of essential hypertension.

V. REQUIEM FOR THE RANDOM CHAIN NETWORK THEORY OF ENTROPIC PROTEIN ELASTICITY

One of the purposes of the above limited enumeration of the possible roles of entropic elastomeric force in protein structure and funciton is to demonstrate the reasoning that becomes possible once the shackles of the classical theory of rubber elasticity (requiring as it does random chain networks) are removed from consideration of entropic protein elasticity. Useful approaches of three decades ago should give way to more accurate descriptions, made possible by improvements in physical methods and their interpretation. These more correct descriptions can lead to new contributions, to new concepts of mechanism that can be tested by a wide range of experimental approaches. It is pernicious to hold that polypeptide backbone motions of the order of nanoseconds can only be achieved by random chain networks. It is contrary to progress in understanding protein structure and function to assume that the only examples of ordered polypeptide states are α-helix, β-sheet and triple standed helix and that all else is random. It is particuarly curious to see protein structure deduced on the basis of a theoretical approach that has found it necessary to invoke phantom chains that occupy no space and that can pass through one another (70). Once the random chain network theory of entropic protein elasticity is set aside, progress in understanding many fundamental processes utilizing entropic protein elasticity can more readily occur.

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FIGURE LEGENDS

Figure 1 Thermoelasticity Studies: Temperature Dependence of Elastomeric Force at Fixed Extension.
A. Polypentapeptide of elastin cross-linked by 20 Mrads of γ-irradiation while in the coacervate state which is obtained by raising the temperature of solutions of polypentapeptide plus water from 20°C to 40°C to form a dense viscoelastic phase that is 62% water, 38% peptide by weight. The sample is extended to 60% at 40°C and then the force is measured as a function of temperature. In going from 20° to 40°C there is an abrupt development of elastomeric force, but above 40°C the plot of ln[force/T(°K)] versus temperature exhibits a near zero slope. Since the slope is proportional to the f_e/f ratio and since this is near zero, it can be argued that the polypentapeptide of elastin exhibits dominantly entropic elastomeric force in the temperature range above 40°C. The development of elastomeric force in the 20° to 40°C range correlates with an inverse temperature observed by numerous physical methods and seen to be a process of self-assembly into fibers. Therefore the polypentapeptide of elastin is an ansiotropic, entropic elastomer.
B. Ligamentum nuchae elastin exhibits a similar development of elastomeric force on raising the temperature over a somewhat broader temperature range, but at higher temperatures the slope approaches zero and a dominantly entropic elastomer has been concluded. This conclusion is assisted by carrying out the study in 30% ethylene glycol in water which shifts the transition to lower temperature giving a wider temperature range where the slope is near zero. In both cases there is plotted on the right-hand side the temperature profile for aggregation, actually for fiber formation as observed by microscopy, for the constituent peptide on protein.
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Figure 2 Probability distribution, W(r), of chain end-to-end lengths r in nm. The solid line gives the distribution for a freely jointed chain with 10,000 segments of 0.25 nm each (22). This is a random distribution of end-to-end lengths representing the highest entropy state. On stretching of a bulk cross-linked matrix of such a collection of chains, the distribution is displaced from that of a random chain network. The decrease in entropy provides a resistance to deformation and a restoring force. This is a description of the classical theory of rubber elasticity. The dashed curve represents a possible distribution of chain end-to-end lengths where the chains are nearly the same length. In this case an entropic restoring force can derive from the damping of internal chain dynamics on extension. This has been referred to as the librational entropy mechanism of elasticity which as represented, can occur with anisotropic fibrillar elastomers.

Figure 3 Proposed Conformation of the Elastomeric State of the Polypentapeptide of Elastin:
A. β-turn perspective showing the ten atom hydrogen bonded ring which utilizes the $Val^1C\text{-}O\cdots HN\ Val^4$ hydrogen bond. This conformation was first developed in solution using NMR methods and then demonstrated in the crystal for the cyclopentadecapeptide which was shown to be the cyclic conformational correlate of the polypentapep-

tide of elastin. Reproduced with permission from 34.
B. and C. Schematic representations of the helical state (β-spiral) of the polypentapeptide of elastin which is the elastomeric state. In C. the β-turns are included showing them to function as spacers with hydrophobic contacts between the turns of the spiral. Reproduced with permission from 31.
D. Detailed stereo pair of the spiral axis view showing space for water within the β-spiral and showing suspended segments between the β-turn. The suspended segment runs from the α-carbon of Val^4 to the α-carbon of Val^1 and is referred to as the Val^4-Gly^5-Val^1 suspended segment. It is within the segment where the large amplitude, low frequency librational motions are most pronounced (see Figure 4 and 5). Reproduced with permission from 31.
E. Stereo pair of the side view of the β-spiral of the polypentapeptide of elastin. This is one of a family of closely related β-spirals. Seen here are gaps in the surface of the β-spiral on each side of the suspended segments. The contacts between turns of the spiral utilize the Val and Pro hydrophobic side chains. The structure in E. is displayed the same as in the schematic representation in C. It is the optimization of intramolecular hydrophobic interactions that is responsible for β-spiral formation. Reproduced with permission from 35.
F. and G. Supercoiling of β-spirals to form twisted filaments of dimensions similar to those observed in transmission electron micrographs of negatively stained polypentapeptide, α-elastin and tropoelastin coacervates (14,30,40) and of negatively stained elastin. The structure is given in F. in α-carbon to α-carbon virtual bond representation and in G. in terms of spheres of different sizes centered at the α-carbon locations. Reproduced with permission from 28.

Figure 4 Stereo pair view of a pentadecapeptide segment in the β-spiral conformation of Figure 3E in which the central Val^1 α-carbon to Val^1 α-carbon pentamer has been allowed to assume conformations within a 2 kcal/mole residue cut-off energy. What is observed is a rocking motion of peptide moieties. In the relaxed state in A., large librational motions are observed whereas in an extended state, in B. at 130% extension, the librational amplitudes are markedly damped. This decrease in amplitude of the librations and possibly an associated increase in the frequency of the librational motions on extension is the decrease in entropy that resists elongation and that provides the restoring force. This is called the librational entropy mechanism of elasticity and this mechanism for developing entropic elastomeric force can occur in any polypeptide segment wherein the structure favors librational processes. Reproduced with permission from 29.

Figure 5 Dielectric permittivity (real part) of the polypentapeptide of elastin coacervate which is 38% peptide and 62% water by weight. On raising the temperature from 20° to 40°C there develops an intense, localized, Debye-type relaxation near 20 MHz. As the only dipolar entities are water and peptide moieties and because the intensity of the relaxation is so large and the frequency relatively low with a low temperature dependence, the relaxation is assigned to a peptide librational motion. Because the relaxation is at a localized frequency the polypentapeptides must be developing a regular structure

as the temperature is raised from 20° to 40°C. The development of this relaxation correlates with the development of elastomeric force observed in Figure 1A. The relaxation is taken to be due to the librational motions shown in Figure 4A. Reproduced with permission from 36.

Figure 6 Dielectric permittivity (real part) of the coacervate state of α-elastin which is a 70,000 molecular weight chemical fragmentation product of elastin. Below 15°C there is a monotonically increasing permittivity from several hundred MHz to 1 MHz. But as the temperature is raised there develops a relaxation near 20 MHz. As α-elastin contains the polypentapeptide of elastin which exhibits a similar relaxation, see inset and Figure 5, this relaxation in α-elastin has been assigned to the same or similar peptide librational processes. The development of the relaxation with temperature in the 15° to 45°C temperature range correlates with the development of elastomeric force over the same temperature range as seen in Figure 1B. Thus this along with considerable other data on elastin, α-elastin and tropoelastin including thermal denaturation of elastomeric force and elastic modulus of elastin at 80°C (33) allows the conclusion that elastin too is a non-random entropic elastomer. Reproduced with permission from 41.

Figure 7 Comparison of a series of studies on a related series of elastomeric sequential polypeptides: Ile^1-PPP is $(Ile^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$; PPP is the polypentapeptide of elastin, $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$; and PTP is $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Gly^4)_n$. These are all high polymers with molecular weights greater than 50,000 daltons. In A. are the temperature profiles for aggregation which have been shown to be temperature profiles for fiber formation, that is, fiber formation occurs by an inverse temperature transition utlizing intermolecular hydrophobic interactions. Increasing the hydrophobicity of the repeating unit as in Ile^1-PPP causes the transition, i.e. fiber formation, to occur at lower temperature than for PPP; Ile is more hydrophobic than Val. Decreasing the hydrophobicity of the repeating unit as in PTP causes the aggregations, i.e. fiber formations, to occur at higher temperature.

In B. the conformation of each of the sequential polypeptides is followed by circular dichroism of suspensions where in the concentration was kept low enough so that the particulate distortions due to the small suspended aggregates were not significant. Observed in each case is an increase in intramolecular order as the temperature is raised through the transition.

In C. the temperature dependence of elastomeric force when the γ-irradiation cross-linked coacervates are set at a fixed extension is followed. The development of elastomeric force is found to have shifted to the temperature range of the inverse temperature transition. This is a clear demonstration that elastomeric force develops as the result of an inverse temperature transition dependent on the hydrophobicity of the polypeptide. The elastomeric state is the more-ordered state and loss of elastomeric force can be achieved by decreasing order. The temperature range of the inverse temperature transition can be shifted by changing hydrophobicity of the polypeptide. If the temperature range of the transition could be reversibly shifted at body temperature then elastomeric force could be turned on and off. Adapted with permission from 55 and 56.

Figure 8 Effect of Inverse Temperature Transition on the Length of the Elastomer. On raising the temperature from 20° to 40° the 20 MRad γ-irradiation cross-linked polypentapeptide of elastin, X^{20}-PPP, undergoes a dramatic shortening to 40% of its 20°C length. This study is carried out at zero load (zero force). The structuring that occurs during the inverse temperature transition to form the β-spiral type of structure results in a shortening of the strip of X^{20}-PPP. A similar but less dramatic and more gradual shortening is observed for bovine ligamentum nuchae elastin. Typical of rubbers, latex expands on raising the temperature. Thus elastomeric force is lost in part due to the structural transition. If by making the polypeptide less hydrophobic, the transition temperature range should shift to higher temperature and the elastomer would at body temperature lengthen and release or relax the force between two contact points. Reproduced with permission from 59.

Figure 9 Prolyl hydroxylation of the polypentapeptide of elastin by the enzyme prolyl hydroxylase decreases the hydrophobicity of the polypeptide and shifts the temperature range for the inverse temperature transition 10°C to higher temperatures. Using synthetic polypentapeptide in which 10% of the pentamers contained hydroxyproline instead of proline causes a similar shift. Of the order of one hydroxyl introduced in 50 residues causes a substantial shift in the transition, as much as 10°C. Considered in terms of Figure 7C., this would shift the development of elastomeric force to a higher temperature. Considered in terms of Figure 8, this prolyl hydroxylation would at 37°C result in a lengthening of the elastomer. Thus an enzymatic modification is expected to cause a relaxation of elastomeric force at body temperature. If the enzymatic modification were phosphorylation and dephosphorylation then entropic elastomeric force could be turned off and on as desired for changing structural states in connective tissue and elastomeric components of muscle or for changing the functional state of an enzyme or channel, for example.

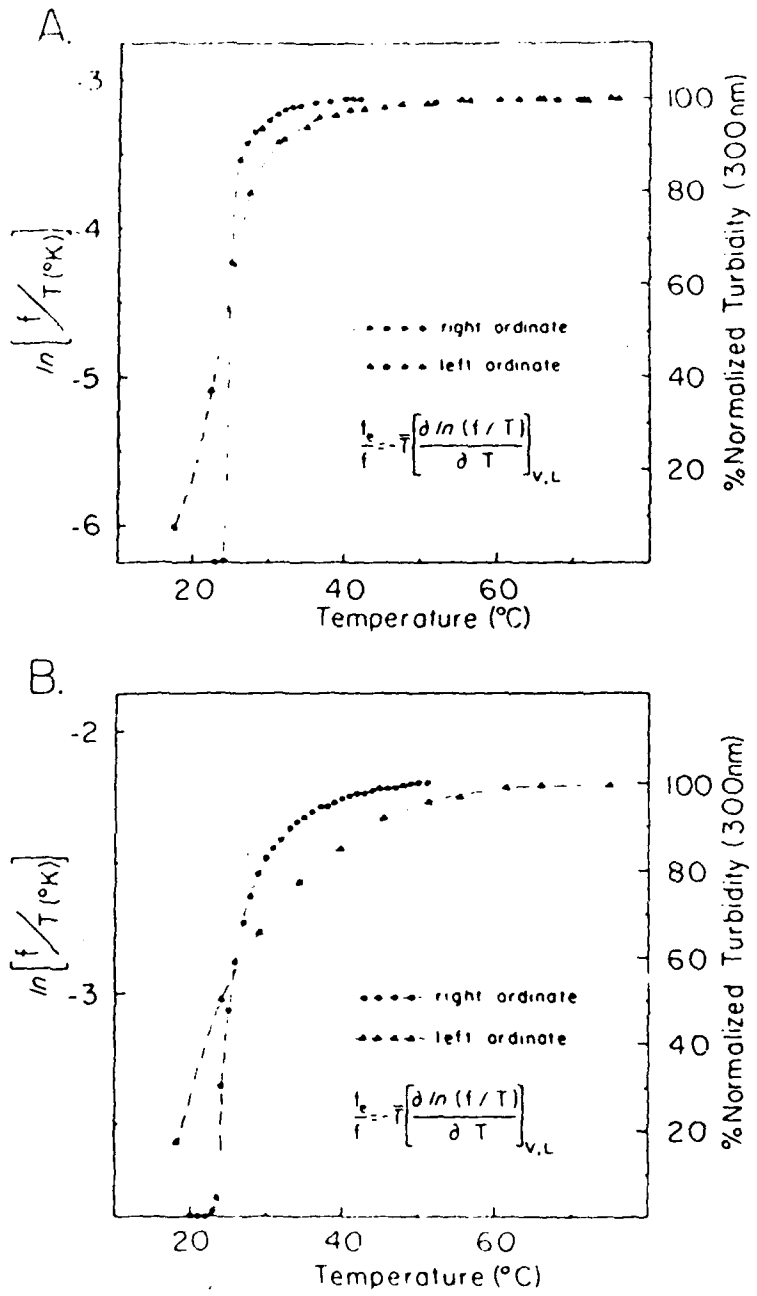

A.
-3
-4
-5
-6
ln [f/T (°K)]
100
80
60
40
20
% Normalized Turbidity (300nm)
right ordinate
left ordinate
$\frac{f_e}{f} = -\overline{f}\left[\frac{\partial \ln (f/T)}{\partial T}\right]_{V,L}$
20
40
60
Temperature (°C)
B.
-2
-3
ln [f/T (°K)]
100
80
60
40
20
% Normalized Turbidity (300nm)
right ordinate
left ordinate
$\frac{f_e}{f} = -\overline{f}\left[\frac{\partial \ln (f/T)}{\partial T}\right]_{V,L}$
20
40
60
Temperature (°C)

FIGURE 1

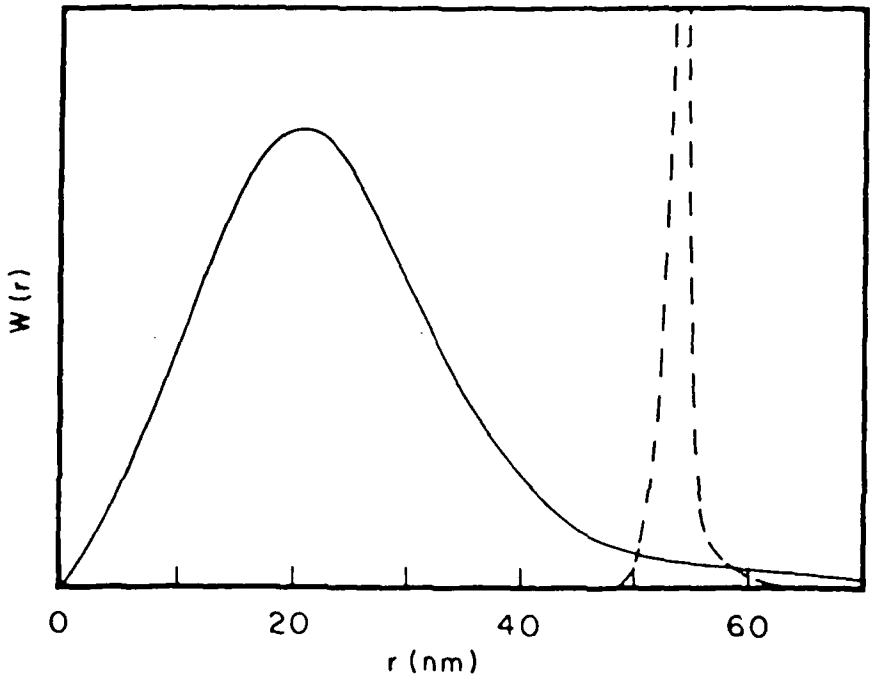


FIGURE 2

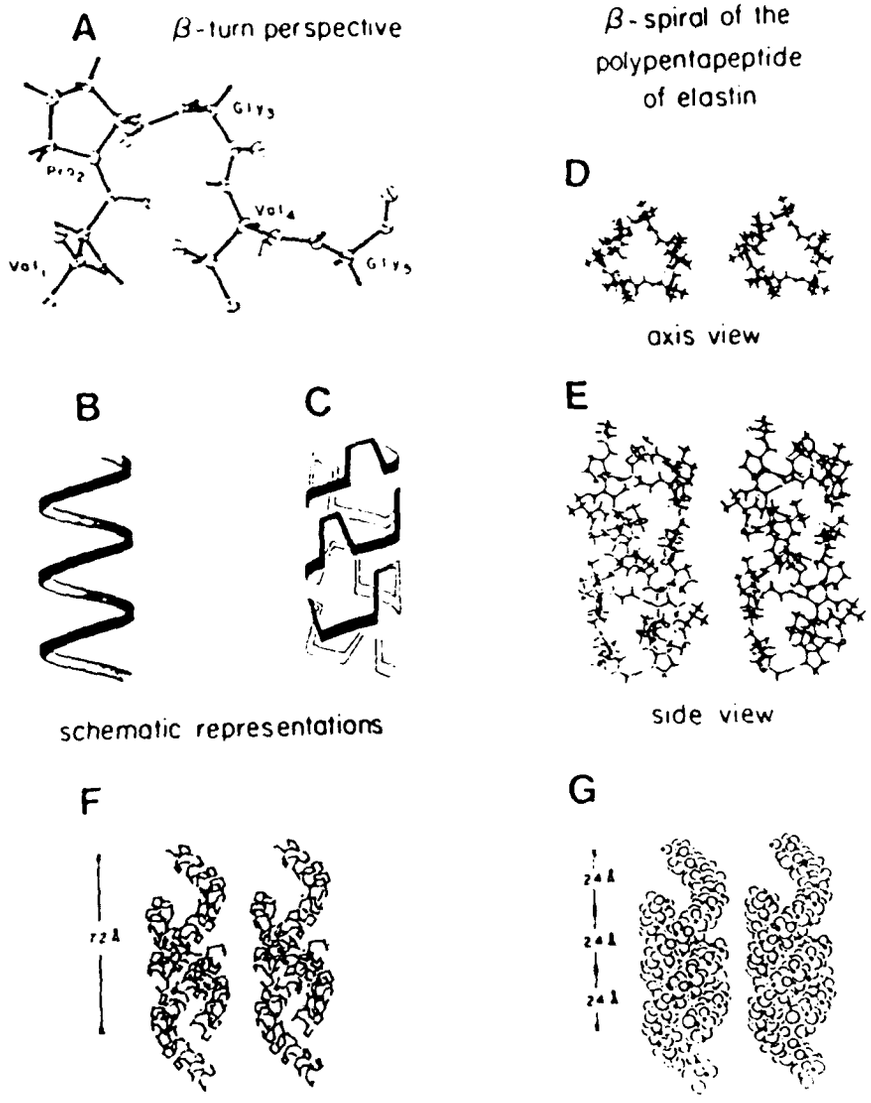

A
β-turn perspective
β-spiral of the polypentapeptide of elastin
Gly3
Pro2
Val4
Val1
Gly5
D
axis view
B
C
E
schematic representations
side view
F
G
72 Å
24 Å
24 Å
24 Å
twisted filament (super coiled) representations

FIGURE 3

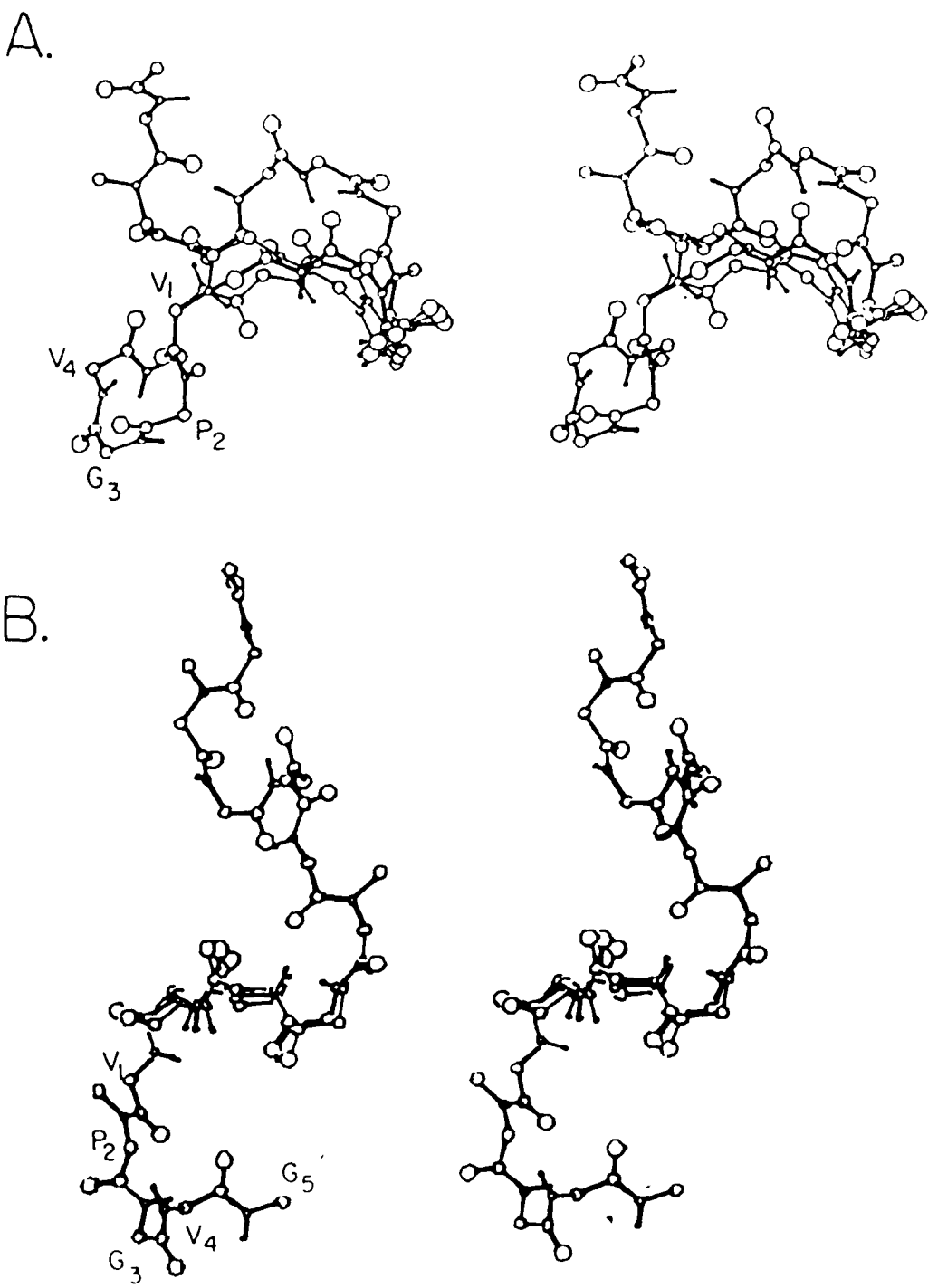
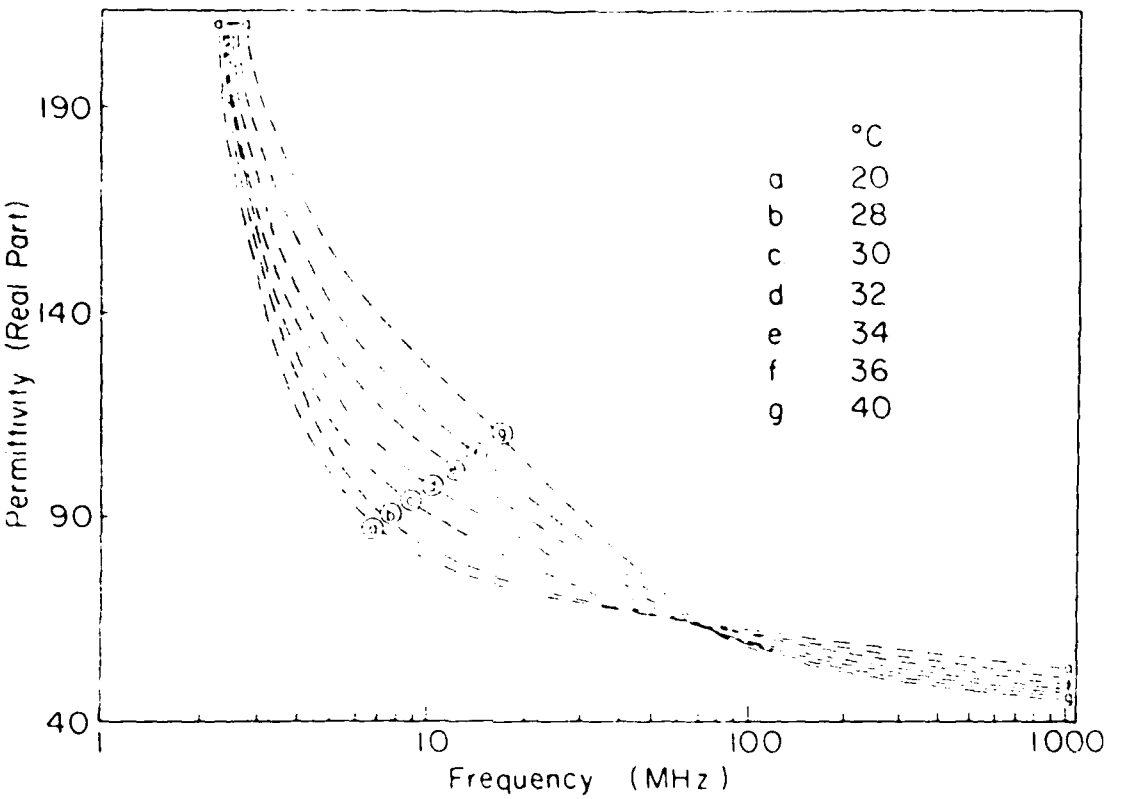


FIGURE 4


190
140
90
40
Permittivity (Real Part)
1
10
100
1000
Frequency (MHz)
°C
a 20
b 28
c 30
d 32
e 34
f 36
g 40

TEMPERATURE DEPENDENCE
DIELECTRIC RELAXATION SPECTRUM OF
α-ELASTIN COACERVATE

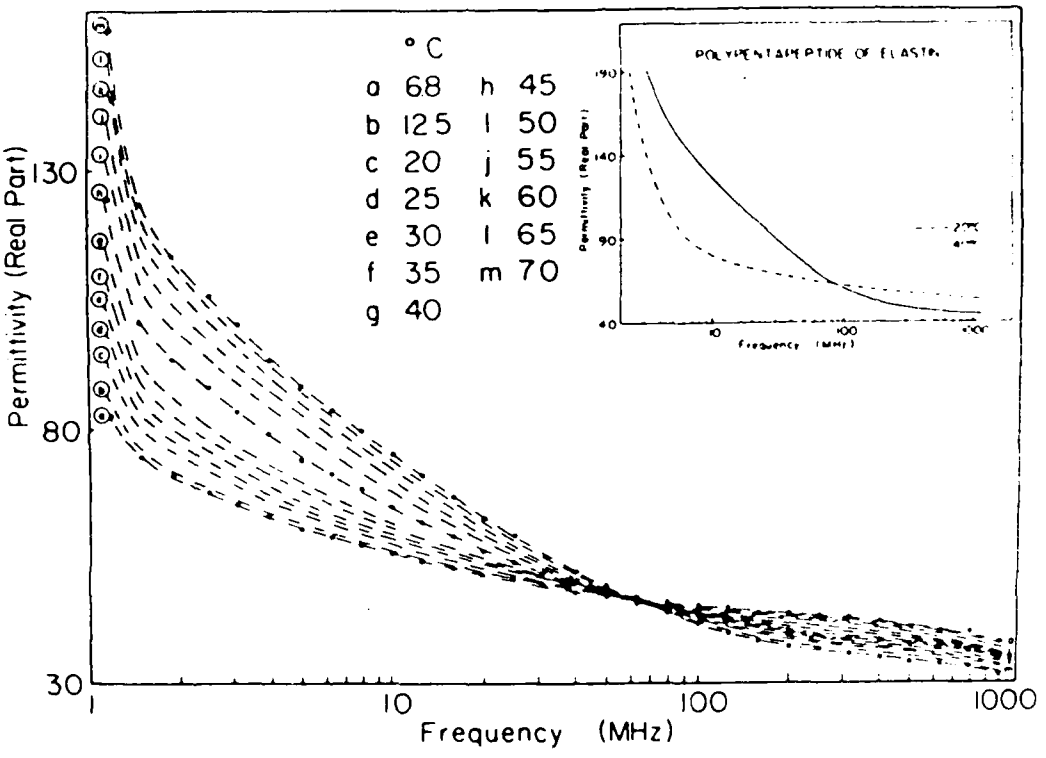


FIGURE 6

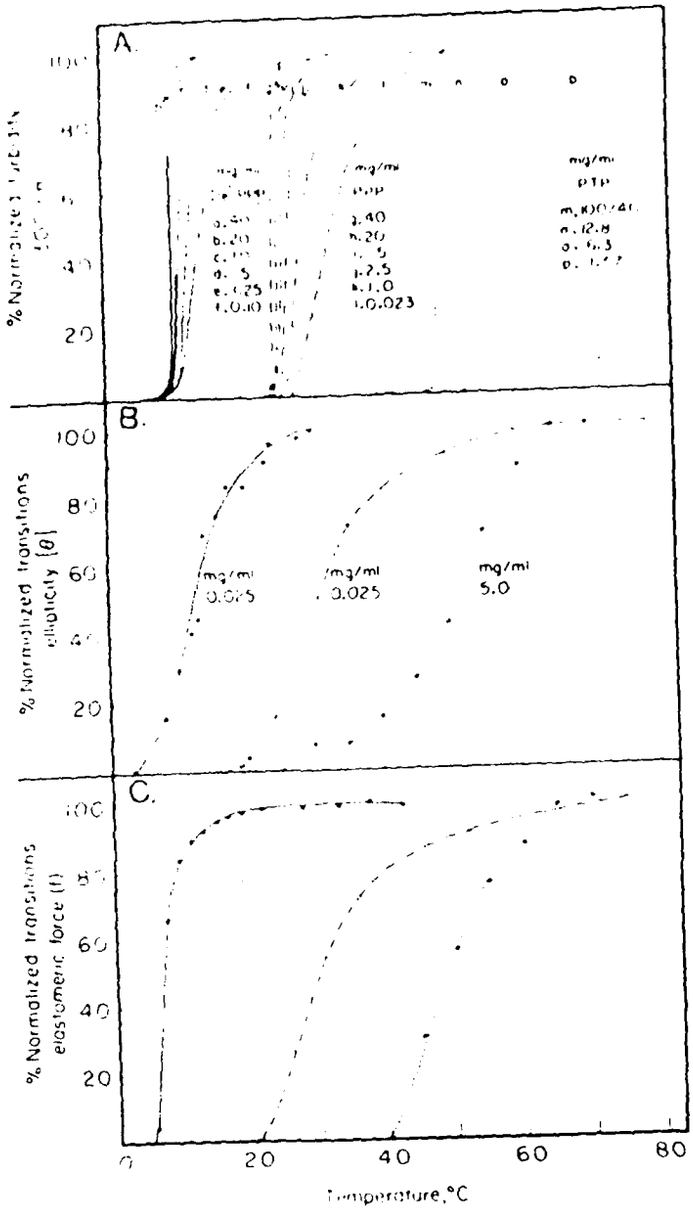

A.
B.
C.
% Normalized transitions ellipticity [θ]
% Normalized transitions elastomeric force (f)
20
40
60
80
100
mg/ml
5.0
Temperature, °C

FIGURE 7

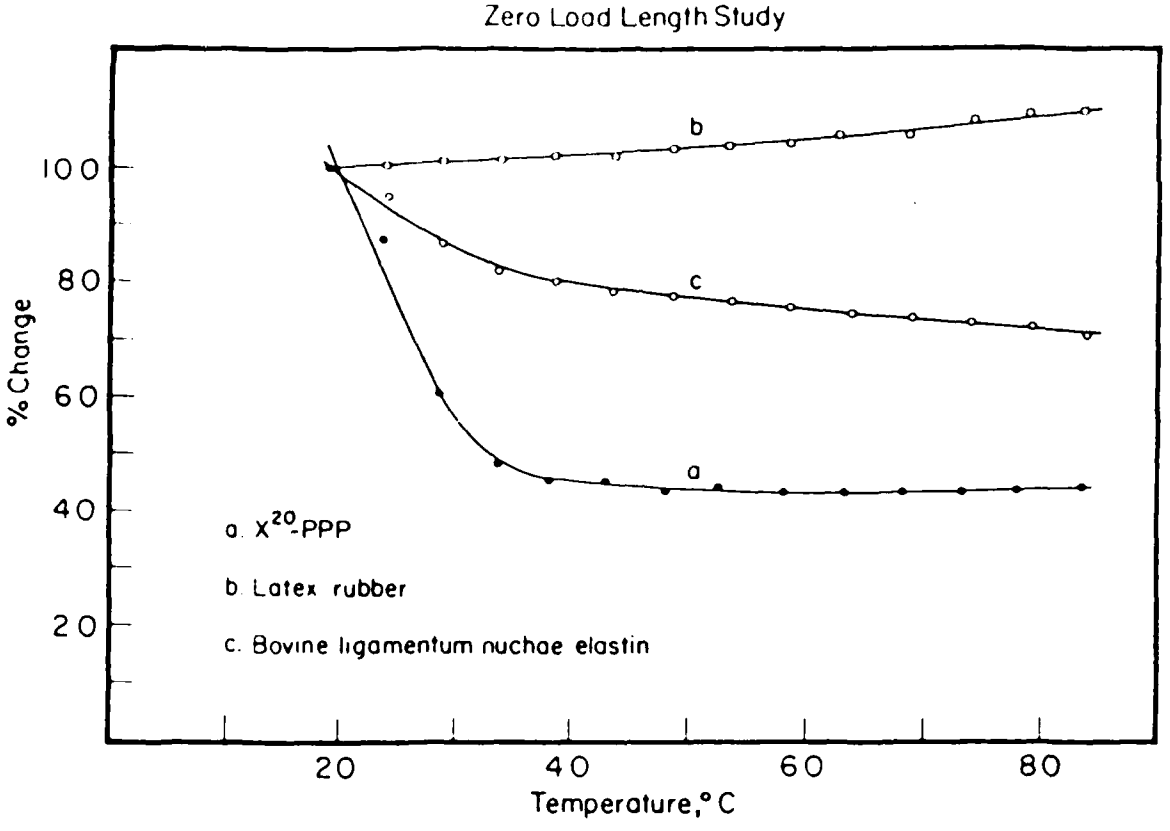

Zero Load Length Study
100
80
60
40
20
% Change
b
c
a
a. X20-PPP
b. Latex rubber
c. Bovine ligamentum nuchae elastin
20
40
60
80
Temperature, °C

FIGURE 8

Temperature Profile of Coacervation (1mg/ml)
Effect of Prolyl Hydroxylation in HCO-$(Val_1-{}^{*}Pro_2-Gly_3-Val_4-Gly_5)_n$-Val-OMe

a. 0% Hyp
b. 1% Hyp
c. 10% Hyp
d. 100% Hyp
e. enzymatically hydroxylated

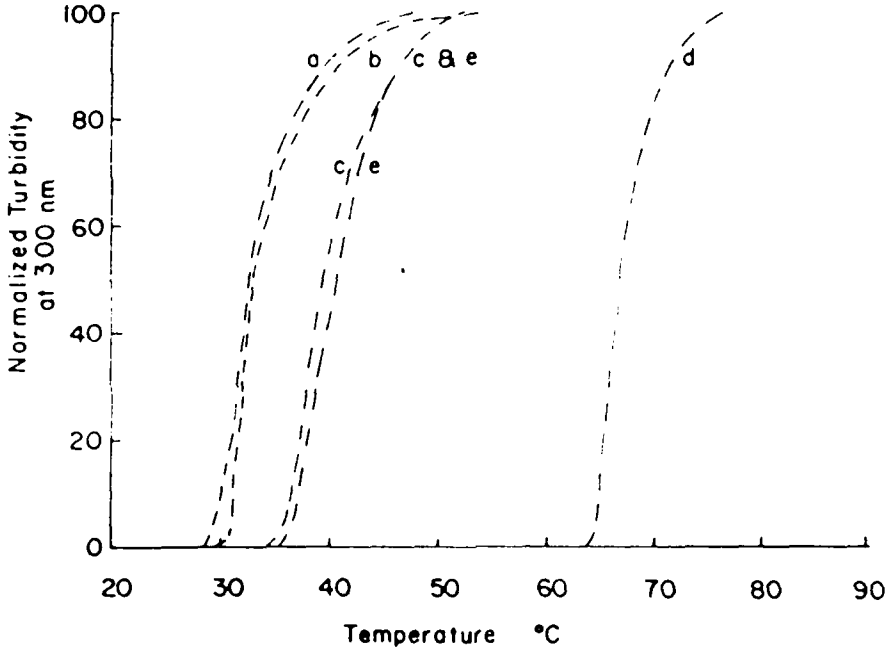
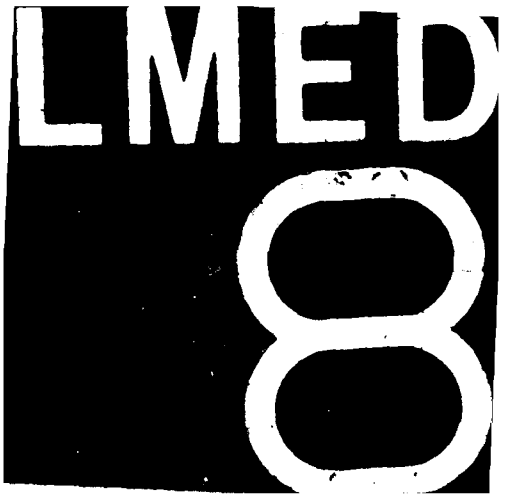


FIGURE 9


LMED
8